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PRINCIPAL INVESTIGATOR: Asok Banerjee, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health Science Center at San Antonio
San Antonio, Texas 78229-3900

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Asok Banerjee, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas Health
Science Center at San Antonio
San Antonio, Texas 78229-3900
Email - asokb@worldnet.att.net

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13. ABSTRACT ***(Maximum 200 Words)***

Tubulin, the αβ dimeric protein of microtubules, bind to different antitumor drugs which are routinely used for cancer chemotherapy. Both α– and β–tubulin exist as 7-8 different isoforms which are expressed differently in different tissues, and also undergo various post-translational modifications including tyrosination-detyrosination, acetylation, poly-glutamylation, polyglycylation and phosphorylation. It is not known whether breast cancer cells differ in the tubulin isoform level or their post-translational modifications. The long-term goal of this project will be to find new prognostic markers for an early detection of breast cancer and to develop alternative therapies against tbreast cancer. The primary goal is to study the expression of different forms of tubulin and their post-translational modifications in human breast cancer cells. Our previous results showed that paclitaxel resistant breast cancer cells express β_{III} isoform selectively. Thus, it was felt necessary to make a full length cDNA of β_{III} isoform for overexpression in breast cancer cells. Here we report the preparation of the cDNA and its overexpression in breast cancer cells. Stable MCF-7 cell lines overexpressing β_{III} isoform are found to be more resistant to paclitaxel when compared with the wild-type MCF-7 cells.

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A Bongiorni May 30, 2001

PI - Signature Date

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Introduction

Tubulin, the heterodimeric αβ subunit of microtubules, exist as multiple isoforms whose expression pattern differ from one tissue to the other (1-4). In mammalian system, there are about 5-7 different isoforms of α– and β– tubulin (5-13). Both α– and β–tubulin also undergo a variety of post-translational modifications (14-25); α–tubulin undergoes tyrosination-detyrosination at the C-terminus and acetylation at Lys^{40} (20,21); β_{III}–tubulin undergoes phosphorylation at a Ser residue (23-25); both α– and β–tubulin also undergo polyglutamylation and polyglycylation, in which glutamyl or glycyl units are attached as side chains through the γ–carboxyl of a Glu residue near the carboxy terminal (15-19). We have previously prepared monoclonal antibodies to different tubulin isoforms, and also purified some of the isoforms from bovine brain (26-40). Isotypically pure different tubulin dimers have been found to differ in their assembly, conformation, and binding to antitumor drugs (26-40).

The primary aim of this project is to study the expression of different tubulin isoforms and their post-translational modifications in breast cancer cells. The idea is to see whether there is any difference in breast cancer tubulin that can be utilized to discover prognostic markers or novel targets for the treatment of breast cancer. The project is on its final year, and has so far opened up new lines of research that was not present in the original proposal.

Expression of tubulin isoforms in human breast cancer cells resistant to paclitaxel:

Preparation of drug-resistant Breast Cancer Cells:

It has been reported that certain tubulin isoforms get expressed when cancer cells become resistant to anticancer drugs. To study the tubulin isoforms we prepared breast cancer cells resistant to antimitotic drugs. The cell lines were prepared by initially growing breast cancer cell lines MCF-7 and MDA-MB-231 in the presence of 1 nM of colchicine, podophyllotoxin, vinblastine or paclitaxel. Verapamil was kept in the growth medium to exclude multidrug-resistant cells. The drug concentration was gradually increased by 1.5 times. After 3-4 months of selection, two drug-resistant lines **MCF7/PTX20** (resistant

to paclitaxel) and **MDA-MB-231/POD60** (resistant to podophyllotoxin) were obtained.

Immunoblot analysis of β–tubulin isoforms in drug-resistant breast cancer cells:

The drug-resistant breast cancer cells were grown to confluency in T-150 culture flasks. The cells were trypsinized and harvested. The cell extract was mixed with equal volume of 2X Laemmli sample buffer, boiled for 5 min, and was analyzed by SDS-polyacrylamide gel electrophoresis and immunobloting using monoclonal antibodies to β_{II}, β_{III}, and β_{IV}. The paclitaxel-resistant MCF-7 cells contains much higher amounts of β_{II} and β_{III} as compared to the drug-sensitive wild type cells. The amount of β_{IV} was marginally increased in the resistant cells. On the other hand, podophyllotoxin-resistant cells exhibited a decrease in the content of all three isoforms β_{II}, β_{III}, and β_{IV}. Since no antibody was available, it was not possible to see the status of the other β–tubulin isoforms by immunoblotting. **At this point it is not clear why the level of some of the isoforms gets elevated while that of others decrease. It may be possible that the cells can identify those isoforms that have the lowest interaction with the drug, and specifically overexpress those isoforms, while the isoforms that have the highest affinity for the drug get lower expression.**

Preparation of cDNA constructs for the expression of GFP-tagged β_{III} tubulin in human cancer cells

In an effort to overexpress individual tubulin isoforms, I have been working on the cloning of full length cDNA specific for individual tubulin isoforms. By using primers specific for β_{III} tubulin, I have prepared full length 1353 bp cDNA from total RNA isolated from MCF-7 cells by RT-PCR using AMV reverse transcriptase and taq DNA polymerase. The product showed a single 1353 bp band in 1.5% agarose gel.

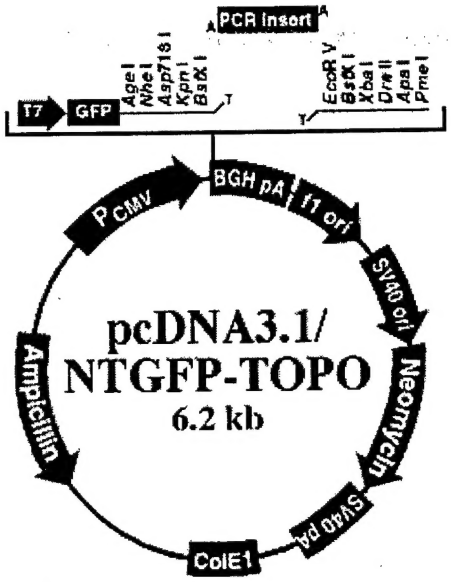
Primers for cloning full-length cDNA for β_{III} tubulin (Human):

Forward: **5'- ATG CGG GAG ATC GTG CAC ATC -3' (+1- 21)**

Reverse: **5'- TCA CTT GGG GCC CTG GGC CTC-3' (+1330-1353)**

The PCR product was inserted in the following mammalian expression vector PC DNA 3.1 GFPNT TOPO (obtained from Invitrogen) at the multiple

cloning site in a 5 minute ligation process and was used to transform *E. coli Top 10* competent cells.



The vector has an **ampicilin resistant gene** for the selection of bacteria and a **neomycin resistant gene** for the selection of stable transfectants for mammalian expression. The transformed mixture was plated on an LB agar plate containing ampicillin and was incubated overnight at 37^0C. About 10 -15 colonies were picked, grown in LB broth overnight, and the plasmids were prepared by a miniprep procedure using a Qiagen kit. The plasmids were tested for the insert size as well as the orientation by restriction digestion followed by agarose gel electrophoresis (since the vector can integrate the DNA in both orientation). The insert size was checked by a double digestion using *Kpn* I and *Eco*R V. The orientation was checked by a double digestion with *Kpn* I and *Bam*H I. Since the β_{III} cDNA sequence (1350 bp) has a *BamH* I site at the base 1032, the plasmid with the right orientation yields a fragment of 1050 bp while the plasmid with a wrong orientation yields a fragment of 350 bp. After selecting the correct plasmid the sequence was confirmed by DNA sequencing. The sequence matched almost perfectly with that of the published sequence of human β_{III} tubulin.

Overexpression of GFP-tagged β_{III} tubulin in human breast and ovarian cancer cells

The β_{III} plasmid was constructed as above at the 3' end of a GFP open reading frame under the influence of a CMV promoter so that GFP is tagged at N-terminal of β_{III} tubulin. Human breast cancer cells (MCF-7) and the ovarian cancer cells (SK-OV-3) were transfected with the GFPNT-β_{III} plasmid using the Lipofectamine Plus reagent from GIBCO BRL. The cells were plated on a 12-well plate on the previous day. The transfection was carried out in DMEM without fetal bovine serum for 5 h at 37^0C in a humidified CO_2 incubator. After 5 h, serum was added to the cells. **For transient transfection**, the cells were plated over tissue culture treated glass cover slips. The samples were examined at 24 h, 48h, and 72h, under a fluorescence microscope using a FITC filter set for visualization of green fluorescence.

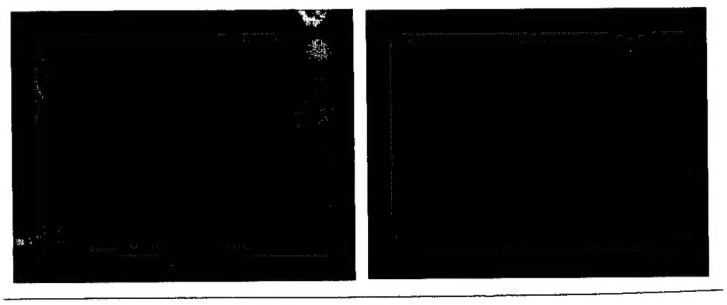


Fig 1

For stable transfection, after 24h of transfection the cells were maintained in the complete medium containing 0.5 mg/ml G 418 (neomycin). After 2-3 weeks, when the colonies started showing up, the cells were given passages to bigger flasks. Fig 1 shows the fluorescence microscopic photographs of stable SK-OV-3 cells overexpressing GFP-tagged β_{III} tubulin (green, left). The photograph on the right shows the immuno-fluorescence staining of the same cells treated with a monoclonal antibody to tyrosinated α–tubulin (AYN 6D.10) followed by an incubation with Cy3-labeled secondary antibody (red, right).

The effect of overexpression of β_{III} tubulin on the paclitaxel sensitivity of SK-OV-3 ovarian cancer cells:

In a preliminary experiment, we have compared the PTX sensitivity of the the wild type SK-OV-3 ovarian cancer cell line and the cells overexpressing GFP-tagged β_{III} tubulin. We find that the IC_{50} value of paclitaxel is increased significantly in cells overexpressing β_{III}. The experiment was carried out on a mass culture, where the expression of β_{III} are not same for all the cells. The experiment need to be repeated with selected clones. These data indicate that an increase in the expression of tubulin may reduce the drug sensitivity. This will be tested in MCF-7 breast cancer cells.

Post-translational modifications in breast cancer tubulin:

Efforts were initiated to study the post-translational modifications in tubulin from breast cancer cells. In a preliminary experiment, cell extracts from **MCF7** and MDA-MB-231 cells were tested for the acetylation and tyrosination status in tubulin. When compared with bovine brain tubulin, both the acetylation and the tyrosination status in breast cancer tubulin seems to be elevated. Since the data are too preliminary and are without the right control cells, the experiments will be repeated. At present, the tubulin is being purified from the breast cancer cells and the purified tubulin will be tested for its post-translational polyglutamylation and polyglycylation by MALDI TOF mass spectrometry.

MALDI TOF mass spectrometry of bovine brain tubulin:

Before doing the MALDI TOF analysis on purified tubulin from the breast cancer cells, we tested the procedure on the tubulin purified from bovine brain. Since it is easily available, we can isolate milligram quantities of purified tubulin.

Separation of α–tubulin fractions:

Bovine brain tubulin contains two α–tubulin isoforms, α1/2 and α4. Using an anti-α–tubulin immunuaffinity column we have previously fractionated bovine brain tubulin into three functionally active forms, fractions A, B, and C (40). Fraction A mainly contains non-tyrosinated forms of α1/2 including Δ2 tubulin. Fraction B is a mixture of the non-tyrosinated forms of α1/2 and α4. Fraction C is essentially the tyrosinated form of α1/2.

Mass spectrometric studies revealed that the fraction C ($\alpha^{Tyr}1/2$)is polyglutamylated with 1-4 Glu residues, tetraglutamylated form being the predominate one. On the other hand, the fraction A is found to contain post-translationally added 1-3 glycine residues.

Key Research Accomplishments

- We have studied the expression of tubulin isoforms in breast cancer cell lines by immunoblotting and RT-PCR analysis.
- Paclitaxel resistant MCF-7/PTX20 cells express increased amounts of β_{II} , β_{III} but not β_{IV}.
- Podophyllotoxin resistant MDA-MB-231 POD60 cells express lower amounts of β_{II} , β_{III}, and β_{IV}.
- We have prepared the full-length cDNA for β_{III} -tubulin from MCF-7 breast cancer cells.
- We have prepared GFP-tagged β_{III} -tubulin construct in the vector PC DNA 3.1 Topo.
- We have overexpressed GFP-tagged β_{III} -tubulin in MCF-7 breast cancer cells and obtained stable cell lines.
- MCF-7 cells overexpressing β_{III} -tubulin are more resistant to paclitaxel than the wild-type MCF-7 cells.
- These results support the hypothesis that tubulin isoform level in a cell line may determine the drug sensitivity. This is a major finding.
- We have performed preliminary MALDI TOF mass spectrometric studies for the identification of post-translational modifications in bovine brain tubulin. We have discovered polyglycylation in brain tubulin. Similar studies will be performed on the tubulin form breast cancer cells.

Reportable Outcomes

1. We have overexpressed GFP-tagged β_{III} -tubulin in MCF-7 breast cancer cells and obtained stable cell lines.

2. We find that the MCF-7 cells overexpressing β_{III} -tubulin are more resistant to paclitaxel than the wild-type MCF-7 cells.

3. Our results support the hypothesis that tubulin isoform level in a cell line may determine the drug sensitivity. This is a major finding.

4. We have performed preliminary MALDI TOF mass spectrometric studies for the identification of post-translational modifications in bovine brain tubulin. We have discovered polyglycylation in brain tubulin.

Publication

Four manuscripts will soon be submitted for publication:

1. Banerjee, A. (2001) Assembly of alpha-tubulin isoforms with different post-translational modifications.
   Manuscript to be submitted to J. Biol. Chem.

2. Banerjee, A. (2001) MALDI TOF mass spectrometric evidence for the presence of post-translational glycylation in bovine brain tubulin.
   Manuscript to be submitted to J. Biol. Chem.

3. Banerjee, A. (2001) Expression of β–tubulin isoforms in podophyllotoxin -resistant human breast cancer cells.
   Manuscript to be submitted to J. Biol. Chem.

4. Banerjee, A. (2001) Overexpression of β_{III} tubulin in MCF-7 breast cancer cells increases resistance to paclitaxel.
   Manuscript to be submitted to J. Biol. Chem.

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